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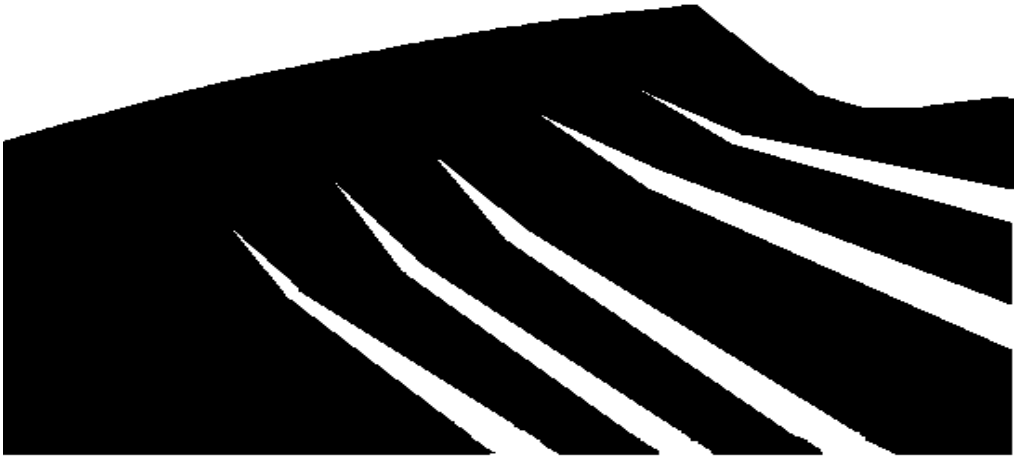
USE OF A FLOW CYTOMETER TO DETERMINE PARTICLE CONCENTRATIONS IN SOLUTION

LOS ALAMOS

QUALITY PROGRAM



APPROVAL FOR RELEASE

P. W. REIMUS - PREPARER Signature on file	DATE Date on file
P. W. REIMUS - PRINCIPAL INVESTIGATOR Signature on file	DATE Date on file
M. J. CLEVENGER - QUALITY ASSURANCE PROJECT LEADER Signature on file	DATE Date on file

Los Alamos
Yucca Mountain Site Characterization Project

HISTORY OF REVISION

REVISION NO.	EFFECTIVE DATE	PAGES REVISED	REASON FOR CHANGE
R0	01/18/96	N/A	This procedure supersedes LANL-EES-15-DP-327.
R1	03/21/97	4, 5, 6, 7, 16, & 17	Minor non-substantive editorial changes.

USE OF A FLOW CYTOMETER TO DETERMINE PARTICLE CONCENTRATIONS IN SOLUTION

1.0 PURPOSE

This detailed procedure (DP) describes the use of a flow cytometer to determine concentrations of fluorescent particles in aqueous solutions. It also describes the use of a flow cytometer to determine concentrations of non-fluorescent particles in aqueous solutions, although there are greater restrictions and less accuracy for non-fluorescent particles than for fluorescent particles. This DP is intended to support the C-Wells Reactive Tracer Studies for the Yucca Mountain Project (YMP), although it could also be used for other YMP studies.

2.0 SCOPE

This DP applies to the use of a flow cytometer to determine concentrations of fluorescent particles and/or non-fluorescent particles in aqueous solutions. It does not apply to configuring a flow cytometer to make these measurements, as this must be done by a trained specialist. It also does not apply to running the data acquisition/reduction software associated with the flow cytometer. However, this DP does describe how the YMP user can determine whether the flow cytometer configuration is adequate and whether minor alignment adjustments are needed as measurements are being made.

3.0 REFERENCES

Horan, P. K. and L. L. Wheeless, Jr. "Quantitative Single Cell Analysis and Sorting," Science, Vol. 198, pp. 149-157, Oct. 1977.

Sklar, L. A. "National Laboratory Provides Laser Resource in Cytometry," Laser Focus World, Aug. 1991.

Steinkamp, J. A., R. C. Habbersett, and R. D. Hiebert. "Improved Multilaser/Multiparameter Flow Cytometer for Analysis and Sorting of Cells and Particles," Rev. Sci. Instrum., 62(11), pp. 2751-2764, Nov. 1991.

LANL-YMP-QP-02.5, Selection of Personnel

LANL-YMP-QP-02.7, Personnel Training

LANL-YMP-QP-03.5, Documenting Scientific Investigations

LANL-YMP-QP-08.1, Identification and Control of Samples

LANL-YMP-QP-12.3, Control of Measuring and Test Equipment and Standards

LANL-YMP-QP-17.6, Records Management

4.0 DEFINITIONS

4.1 Flow Cytometer

An instrument in which a solution containing particles up to several microns in diameter is "hydrodynamically focused" through one or two orifices and fluid sheaths into a narrow stream (less than 100 μm diameter) that passes through

one or more laser beams. Besides creating a very uniform stream of circular cross-section, the hydrodynamic focusing is intended to “line up” the particles in single file as they pass through the laser beam(s). The laser beams can be chosen and filtered to selectively excite fluorescent dyes of various excitation wavelengths (with which the particles can be tagged), and a bank of photomultipliers equipped with additional light filters can be used to selectively detect fluorescence emission at different wavelengths at a 90 degree angle to the incident laser beam(s). Thus, particles that are tagged with different fluorescent dyes can be readily distinguished and counted separately from non-fluorescent particles and from each other (even when they are in the same sample). Photomultipliers can also be used to detect forward and 90 degree angle light-scattering caused by the particles as they pass through the laser beam(s). System hardware and software are used to acquire, manipulate, and reduce the data. In this DP, the term “flow cytometer” refers to all the hardware associated with determining particle concentrations with the exception of the computer used for data analysis/reduction and the balance used for weight measurements. Additional information on the instrumentation and capabilities of flow cytometers is contained in the first three references listed in Section 3.0.

4.2 Flow Cytometer Specialist

A person who is very familiar with the operation of flow cytometers and is capable of configuring them specifically for measurements described in this DP. The Flow Cytometer Specialist can be a Limited Function Employee, as defined in QP-02.5.

4.3 Test Sample

A sample used to configure a flow cytometer and to run certain tests that ensure correct and accurate flow cytometer operation. The sample should contain all the particles to be analyzed, preferable dispersed in the same water as the experimental samples with unknown particle concentrations (unknowns). The particle concentrations in the test sample should be at least as high as the largest concentration expected in the unknowns.

5.0 RESPONSIBILITIES

The following personnel are responsible for the activities identified in Section 6.0 of this procedure.

- Principal Investigator (PI)
- Procedure User

6.0 PROCEDURE

The use of this procedure must be controlled as follows:

- If this procedure cannot be implemented as written, YMP personnel should notify appropriate supervision. If it is determined that a portion of the work cannot be accomplished as described in this DP, or would result in an undesirable situation, that portion of the work will be stopped and not resumed until this procedure is modified or replaced by a new document, or until the current work practice is documented in accordance with QP-03.5, subsection 6.1.6.

- Employees may use copies of this procedure printed from the controlled document electronic file; however, employees are responsible for assuring that the correct revision of this procedure is used.

- When this procedure becomes obsolete or superseded, it must be destroyed or marked “superseded” to ensure that this document is not used to perform work.

6.1 Principle

A flow cytometer can be used to very accurately determine concentrations of fluorescent particles in liquid samples by simply counting the fluorescence signals in a given volume of liquid as it passes through the laser beam(s). The liquid volume analyzed can be determined by weighing the sample before and after the measurement. The concentrations of non-fluorescent particles can be determined by counting scattered-light signals (though generally to less accuracy than fluorescent particle signals).

6.2 Equipment and Hardware/Software

The equipment and hardware/software associated with this DP include a flow cytometer and its supporting hardware and software, and an analytical or top-loading balance. The hardware items that comprise the flow cytometer can include, but are not necessarily limited to, lasers (with appropriate shielding), light filters, photomultiplier tubes, oscilloscopes, a test tube mount for samples, flow tubing and a flow chamber, electronic signal processing equipment, and digital signal counters. The flow cytometer configuration for a given set of measurements will depend on the size of the particles and the fluorescent dyes that they are tagged with. Typically, a separate photomultiplier will be used to detect each different fluorescent dye or scattered-light signal. Depending on the capabilities of the flow cytometer, several different fluorescence signals or scattered-light signals could be simultaneously detected and quantified. The flow cytometer configuration for each set of measurements will be documented using a flow cytometer configuration sheet (Attachment 1) filled out by either the Flow Cytometer Specialist or the DP User (see subsection 6.3).

Typically, a computer system with an assortment of resident computer programs will be electronically interfaced to the flow cytometer for on-line data

analysis and reduction. For this DP, data analysis and reduction involving the computer system / software will usually consist of generating histograms of particle count vs. Signal intensity (hereafter called “signal intensity histograms”).

A top-loading or analytical balance will be used to weigh samples before and after particle counting so that a sample volume can be determined. Weight measurements are to be made using a balance that is calibrated and controlled in accordance with QP-12.3.

6.2.1 Equipment Malfunctions

Most malfunctions of the flow cytometer equipment described above will result in stoppage of data collection or obvious degradation of the data. However, it is possible that gradual misalignment of the flow cytometer optical equipment or a partial plug in the sample stream could cause subtle degradation of the data. Methods of checking for such data degradation are discussed in subsection 6.5.2. Malfunctions of the computer / data acquisition system will result in the inability to analyze and reduce data. Likewise, malfunctions of the balance used for weight measurements will result in the inability to analyze data.

6.2.2 Safety Considerations

The laser beams that constitute a part of the flow cytometer should be properly shielded, or appropriate eye protection should be worn if the beams are not shielded. Appropriate precautions should be taken if the solutions containing the particles to be analyzed contain hazardous constituents (e.g., dissolved species tracers that are hazardous).

6.2.3 Special Handling

Handling of all equipment associated with this DP should be done in accordance with manufacturer’s or vendor’s guidelines. Special handling of equipment or hardware should be considered on a case-by-case basis as the need arises. Any special handling should be documented in a laboratory notebook.

6.3 Preparatory Verification

For a given set of particle sizes and corresponding fluorescent dyes that are to be simultaneously measured, the flow cytometer will be configured to optimize the intensities and the discrimination of the signals associated with the particles. This involves a rather complex “tuning” of the instrument, which includes laser beam alignment, light filtering, and photomultiplier alignment / voltage adjustment, all of which are done as a sample aliquot is allowed to flow through the laser beam(s). The configuration procedure, which is not addressed in this DP, is best performed by the Flow Cytometer Specialist, although the DP User can perform some or all of the steps at the discretion of the Flow Cytometer

Specialist. The configuration for each set of measurements should be documented on a flow cytometer configuration sheet (Attachment 1).

NOTE: The lower size limit for detection and discrimination of particles depends on many factors, such as the concentration and intensity of fluorescent dyes in the particles, the amount and nature of the background or other interfering particles, and the specific capabilities of the flow cytometer being used. The ability to detect and discriminate particles must be determined on a case-by-case basis by evaluating particles in the instrument being used for analysis, preferable with the particles suspended in a sample of the appropriate groundwater.

6.3.1 Hold Points (N / A)

6.3.2 Calibration

A flow cytometer is not "calibrated" by the traditional method of analyzing a series of standards of known concentration and then preparing a "calibration curve" that relates instrument readings to known concentrations. However, a number of quick tests (see below) are conducted prior to each use of the flow cytometer to ensure the accuracy of the particle concentration measurements. In reference to QP-12.3, these tests effectively constitute a "calibration prior to use" of the flow cytometer.

One reason that traditional calibrations are not used with the flow cytometer is that it is very difficult to prepare accurate particle concentration standards. Particle size standards do not work well as concentration standards for a number of reasons, which include (1) the weight or volume percent solids in the dispersion (reported by the manufacturer) is typically accurate to only $\pm$2 to 5%, which means a $\pm$2 to 5% error in the number of particles, (2) the specific number of particles per unit volume will depend on the particle size distribution, which can have a standard deviation of greater than 10% of the mean, and (3) most particle size standards undergo a certain amount of particle aggregation, which depends on particle surface chemistry, solution composition, storage conditions, and aging. Given these considerations, the error associated with preparing particle concentration standards can be expected to be as much as $\pm$10%, which is large compared to the typical precision of other flow cytometer measurements ($\pm$2 to 3%). Another reason that conventional calibration techniques are not used is that, given the proper configuration and measurement conditions, a flow cytometer is capable of providing a direct measurement of particle concentration rather that a reading that must be interpreted using a calibration curve. Also, absolute calibration is not strictly necessary when the concentration measurements are associated with tracer transport experiments or any experiment in which the quantity of interest is the sample concentration relative to some initial concentration or inlet concentration (i.e., the ratio of sample concentration to initial concentration).

After the flow cytometer is configured, a series of quick tests must be conducted to determine (1) whether the digital counters are counting all the particle signals, (2) whether any of the particle signals are being "missed" by the flow cytometer electronics, and (3) whether any corrections have to be made for background signals. These tests should be repeated each time the flow cytometer is configured for a series of measurements. To do these tests, it is necessary to have several ml of a test sample that contains all the particles to be analyzed at concentrations at least as high as the maximum concentrations expected in the unknowns (and preferable dispersed in the same water as the unknowns—see subsection 4.3).

6.3.2.1 Ensuring that all Particle Signals are Counted by Digital Counters

To ensure that the digital counters are counting all the particle signals, the test sample should be run through the flow cell / laser beam(s), and the threshold voltage levels on the counters should be set to a level well below that of the particle signals so that all signals are counted. However, the counter thresholds should not be set so low that spurious noise or background is also counted. A good initial guess of an acceptable threshold can be made by noting the particle signal voltages and the voltages of the noise / background signals on an oscilloscope or on a signal intensity histogram (generated by the computer software). An acceptable threshold is verified by adjusting the threshold voltage slightly and seeing if this affects the particle count rate. With an acceptable threshold voltage, the count per unit volume should not be affected by more than 3 to 5 percent when minor adjustments are made, but when the threshold is too high or too low, the count per unit volume will be affected significantly.

NOTE: The user may opt to count particles by graphically integrating peaks on signal intensity histograms (see subsection 6.5.1.3 and 6.5.1.4) rather than by using digital counters. In this case subsection 6.3.2.1 can be ignored.

6.3.2.2 It is possible that particle signals could be "missed" by the flow cytometer because they are occurring too rapidly for the electronics of the instrument to "keep up with them." To determine whether any particle signals are being "missed," it is best to measure particle concentrations in the test sample at different sample pressures / flow rates (see subsection 6.5 for measurement procedure). The concentrations measured at different flow rates should agree to within 3 to 5 percent. Missed particles will be indicated by a significantly lower particle concentration at higher pressures / flow rates. When

particles are missed, the particle count rates indicated by the readout (signals / second) on the flow cytometer should be noted for later reference. Sample pressures / flow rates should always be kept low enough that the count rate does not exceed values known to result in missed particles. Lost counts due to coincidence of signals can be estimated from nomographs based on stochastic principles.

Another method that can be used to determine if particle signals are being "missed" is to measure the concentration of one or more dilutions of the test sample. The measured particle concentrations should vary linearly with the amount of dilution. That is, the measured concentration of a tenfold dilution should be a factor of ten lower than that in the test sample (within 3 to 5 percent). If the dilution has a significantly higher concentration than expected, it is probably an indication that particle signals are being missed in the more concentrated sample. In this case, the measurements should be repeated at a lower sample pressure / flow rate until the measured concentrations vary linearly with the amount of dilution. Again, particle count rates that correspond to particles being missed should be noted, and these count rates should not be exceeded when measuring samples.

6.3.2.3 Determining the Background Concentrations of Particles

To determine the background concentrations of particles, it is best to simply measure the particle concentrations in a blank, which should be water that, as closely as possible, is the same as the water in the unknowns but without the particles in it. Background counts should be obtained for each photomultiplier / detector and associated electronics that is used for counting particles. The particle concentrations in the blank should then be subtracted from the measured particle concentrations in all the unknowns. Signal intensity histograms associated with each photomultiplier (generated by the computer hardware / software) should be obtained for both the test sample and the blank. Background signals are indicated by the presence of signal intensity "peaks" in the blank histogram(s). By noting the location of these peaks, it will be possible to determine whether the background is changing with time or with sample when the unknowns are measured (a change would be indicated by a larger or smaller peak, a different shaped peak, a peak in a different location, or a new peak). Subsection 6.5.1.3 contains more information on how to use signal intensity histograms to account for background when making particle concentration measurements. Hardcopy printouts of the signal intensity histograms for both the test sample and the blank should be generated and kept as a record.

In addition, computer-based data files can be collected and saved to record the raw data from each sample analyzed.

NOTE: It is possible that particles tagged with different fluorescent dyes can act as "background" for each other. This happens when the fluorescence spectra of two dyes overlap (or when one particle has a much higher dye loading than another particle) so that one dye produces enough fluorescence at the peak wavelength of the other dye that a signal is registered by the photomultiplier detecting the second dye. This situation is generally recognized during configuration of the flow cytometer, and it can be corrected for by using the methods described in subsection 6.5.1.4 (see the Note for that section).

NOTE: The signal intensity histograms for the test sample provide information on the degree of aggregation of the particles. Aggregates are generally indicated by smaller peaks of greater intensity than the main peak associated with single particles. Doublet peaks are quite common, but triplet and higher-order peaks are often not discernable on the histograms. Recording the signals on a logarithmic scale can help quantify the number of aggregated particles recorded as a single event.

6.3.3 Environmental Conditions

There are no special environmental conditions that must be met when operating the flow cytometer. However, it is advisable to try to keep the ambient temperature as constant as possible because small temperature fluctuations can affect the alignment of the optical equipment.

6.4 Control of Samples

Samples are to identified and controlled in accordance with QP-08.1. Samples can be stored in any airtight container that does not compromise the sample by introducing background particles or fluorescence. Samples should not be stored under fluorescent room lights, as this can, over time, reduce the measured fluorescence intensity of the particles. Glass sample containers are generally preferred over plastic containers because particles can be detached / resuspended from glass walls more readily than from plastic. If possible, samples stored for 12 hours or longer prior to analysis on the flow cytometer should be refrigerated at about 4 degrees Celsius to maximize dispersion stability (i.e., minimize particle aggregation). However, samples should not be allowed to freeze, as this typically destroys stability (upon thawing). Samples should be removed from the refrigerator as soon as possible on the day of the analysis so that they can be as close to room temperature as possible when they are analyzed. Immediately

prior to dispensing an aliquot of the sample for measurement on the flow cytometer, the sample container should be agitated vigorously, preferably using both a mechanical shaker and a sonicator bath. Do not use a method that involves opening the sample container and introducing a stirrer or other object (such as a sonifier tip), as this tends to introduce background particles. Mechanical shaking resuspends settled particles and ensures uniform dispersion of the sample. Sonification helps to resuspend particles that have become attached to the walls of the sample container.

6.5 Implementing Procedure

6.5.1 Concentration Measurement

For each sample, the following steps should be followed to measure particle concentrations:

6.5.1.1 Flushing the Dead Volume with Sample

After agitating the sample (see subsection 6.4), dispense 1 to 2 ml of the sample into a test tube that fits onto the flow cytometer. After mounting the tube on the flow cytometer, pressurize the tube sufficiently to force the sample to flow into the flow chamber and ultimately through the laser beam(s). Allow the sample to flow for minute or so at a relatively high flow rate (i.e., higher pressure) to ensure that the dead volume in the system is entirely flushed out and contains only the dispersion to be analyzed. Adequate flushing is indicated by a relatively constant count rate registered on the digital counters. After the dead, volume has been adequately flushed, stop and flow, remove the test tube, and discard the contents. Do not allow the sample to be displaced from the flow cytometer inlet tubing by backflushing of sheath fluid.

6.5.1.2

Dispense 1 of 2 ml of sample into the same test tube used to flush the dead volume, and weigh the test tube and contents on an analytical or top-loading balance. Record the weight in a laboratory notebook. Mount the test tube on the flow cytometer and pressurize it to an acceptable pressure (below any limits established in subsection 6.3.2.2). Record the sample pressure. Before starting the flow, make sure that all digital counters are zeroed and that the data acquisition/reduction software is set up to begin collecting a new batch of data. Start the flow, and allow at least 0.3 ml of sample to flow through the laser beam so that an accurate weight difference can be measured (1 ml or more is preferable if enough sample is available). Stop the flow, remove the test tube, and weigh it again on the balance. Record the weight, the readouts on the digital counters, and print out the signal intensity histograms for each photomultiplier.

NOTE: It is best to run the samples in order from lowest particle concentration to highest concentration so that errors associated with particle holdup in the dead volume from prior samples is minimized. Although the dead volume can be flushed out in a relatively short period of time, it has been observed that a small number of residual particles from previous samples can persist in the system for quite a long time, especially when analyzing small (<1μm) particles. When measuring a very small concentration immediately after a much larger concentration, it is advisable to flush out the system at a high flow rate with particle-free water until the particle count rate has essentially dropped to zero. This may take several minutes.

6.5.1.3 Accounting for Background Particles

If the background particle concentration remains relatively constant during a series of measurements, the best and quickest way to account for the background is to simple measure it once and then subtract if from each of the unknown measurements (as discussed in subsection 6.3.2.3). However, if the background appears to be changing with different samples (as indicated by changing background separately for each sample. This can be done with the help of the data acquisition / reduction software. If a background peak is not overlapping a particle peak, the background can be subtracted out directly by using the system software to determine the number of particles comprising the background peak (by on-line graphical integration). Alternatively, the software can also be used to count only the particles that comprise the peak associated with the particle being measured. Signal to background ratios can also be determined in this way, and this ratio can then be used to determine how many of the total counts are due to signal or background. If the background peak is significantly lower in intensity (voltage) than any of the particle peaks, it may be possible to adjust the counter threshold voltage so as to count the particles of interest but not the background. If the background peak overlaps a particle peak, the method of measuring the background once and subtracting it from each unknown is the only method that can be used.

NOTE: If an individual sample is contaminated with a population of “foreign” particles of unknown origin that result in a distinguishable peak in a signal intensity histogram, the contamination can be subtracted out by graphical integration in the same way as the background (as described above).

6.5.1.4 Determining Concentrations of Non-Fluorescent Particles

Non-fluorescent particles are detected by the flow cytometer only by scattered light signals. As such, it is not possible to achieve the discrimination and separate counting of non-fluorescent particles that is possible with fluorescent particles tagged with different dyes. Therefore, it is very important that the background particle concentrations be carefully measured and subtracted out to obtain the concentrations of the particles of interest. If the non-fluorescent particles result in peaks in the scattered light intensity histograms that are completely separate from peaks associated with other particles in the system (including background particles), it may be possible to obtain particle counts using the digital signal counters or the data acquisition / reduction software. In the latter case, the number of signals associated with the peak(s) of interest is graphically integrated, and this number is taken to be the particle count.

NOTE: The graphical method of counting particles can also be used when two different populations of fluorescent particles give signals on the same photomultiplier. If the two particles are tagged with different fluorescent dyes, generally one of them will be detected separately on another photomultiplier and its count can thus be subtracted out directly. However, if this is not the case, the peaks on the signal intensity histogram can be graphically integrated as described above to obtain individual particle counts. This method can also be used if there are two particles of different sizes that are tagged with the same fluorescent dye. Alternatively, graphical integration can be used to obtain separate counts for singlets, doublets, and higher-order aggregates, if such peaks are apparent on the histograms. In some cases, it is best to analyze the data using two-parameter histograms (scattered light vs. Fluorescence), as the correlated information provides better data discrimination.

6.5.2 Checking for Gradual Data Degradation

It may take several hours or even more than one day to complete a single set of measurements. During this time, the alignment of the optical equipment may drift or one of the orifices in the flow cell may become partially plugged, thus diverting the flow stream out of the center of the laser beam(s). These occurrences can cause a gradual change in the intensity of the signals (voltages) being registered by the photomultipliers (plugging is usually more obvious than optical equipment drift). Such a degradation can be detected in at least two ways. First, concentration measurements of at least one of the more

concentrated samples should be repeated from time to time. If the measurements do not agree to within 3 to 5 percent (agreement can be worse if concentrations are low, particularly in the presence of a significant background), the optical equipment and flow stream alignment should be checked. Another way to check for alignment drift is to compare signal intensity histograms of successive samples. Alignment drift is indicated by a gradual change in the location of a peak or by a change in the shape of a peak on the histograms. An easy way to check for this is to simply lay one histogram printout on top of another and then hold them up so that light shines through them from behind. Differences in peak location or shape will be readily apparent. However, minor differences will not necessarily affect the quality of the data (as long as all the particles are still being counted, which is indicated by good agreement between repeat measurements). If significant drift is indicated, the Flow Cytometer Specialist should be contacted to realign the optical equipment or to unplug the orifice(s). Alternatively, the DP user can perform these tasks at the discretion of the Flow Cytometer Specialist.

6.5.3 Establishing Measurement Precision

Measurement precision should be established by repeating measurements on a given sample several times over the course of all measurements so that a statistical measure of precision (e.g., standard deviation) can be estimated. If sample volumes severely restrict the number of repeat measurements that can be made, then the repeat measurements should be made on the test sample or a dilution of the test sample.

6.5.4 Documentation of Results

The following information should be recorded / documented each time the flow cytometer is configured for a series of measurements:

- a completed flow cytometer configuration sheet (Attachment 1).
- the steps used to set digital counter thresholds and the threshold voltages established.
- the steps used to determine if signals are being “missed” by the flow cytometer, and the maximum count rate (if any) that samples should be run at.
- the steps used to determine background particle concentrations, and what these concentrations are (if any).
- printouts of the signal intensity histograms of the test sample and blank(s) (discussed in subsection 6.3.2.3).

The following information should be recorded for each concentration measurement:

- sample identification.
- weight of sample test tube before and after measurement and the difference in weight.
- unique identification of the measuring and test equipment per QP 12.3.
- pressure and signal count rate at which the sample was run.
- counts registered on each of the digital signal counters, or counts obtained by graphical integration of appropriate histogram peaks. Any changes in background particle concentrations, changes in signal intensities, or changes in peak locations should be noted, and any manipulation of data to obtain counts should be documented.
- printouts of signal intensity histograms.
- calculated particle concentration(s).

6.6 Data Acquisition and Reduction

The intensity of each individual signal on each photomultiplier is acquired and recorded by the computer system hardware/software that is interfaced with the flow cytometer. The software can be used to present this information as histograms of particle count vs. Signal intensity (for each photomultiplier). Particle counts from individual photomultipliers are also recorded using digital signal counters at present signal thresholds.

Sources of error and uncertainty associated with particle concentration measurements are discussed in subsection 6.7.

6.6.1 Calculations

If there is not background or a background that does not change significantly with time, or if the signals being registered by a counter are coming from only one population of particles, the counts recorded on the digital counters are used to calculate particle concentrations, However, if these conditions are not met, particle counts must be determined by graphical integration of the appropriate peaks on the signal intensity histograms. Particle concentrations are calculated by dividing the particle count by the volume of liquid passed through the flow cytometer. The liquid volume is determined by dividing the weight difference of the sample test tube before and after the measurement by the density of the liquid. If there is a background concentration and the particle count is taken from a digital counter, the background concentration must be

subtracted from the total concentration to obtain the concentration of the particles being measured.

6.6.2 Acceptance Criteria

Acceptance criteria cannot be established in this DP because several major variables (e.g., method of counting particles—either by digital counters or by graphical integration of histograms, size / fluorescent intensity of particles, amount and variability of background) can influence the accuracy and precision of the analyses. When particle concentrations are large relative to background, reproducibility of repeat measurements should be within 3 to 5 percent. The PI reviews the data and associated records and determines the acceptability of the generated data. The PI may reject measurements for any of the following reasons:

- anomalous results,
- unacceptably large standard deviations associated with repeat concentration measurements on the same sample, indicating poor measurement precision,
- operational deviations which call into question the accuracy of the results, and
- inadequate record keeping.

The identity of the rejected results and the basis for rejection are recorded in a laboratory notebook.

6.7 Potential Sources of Error and Uncertainty

Potential sources of error and uncertainty associated with this DP include:

- incorrect or poorly chosen digital counter threshold voltage.
- a sample pressure / flow rate that is too high, resulting in particles being "missed" by the flow cytometer electronics.
- changes in background particle concentrations from sample to sample.
- optical equipment alignment drift or partial plugging of the sample stream, which can cause changes in the intensity of the signals registered by the photomultipliers.
- variability in the amount of liquid held up on the outside of the tubing that draws liquid from the sample test tube, which can cause variability in the weight difference measurements.

The first two sources of error can be minimized by following the "calibration" procedures described in subsection 6.3.2. Accounting for background particle concentrations is discussed in subsections 6.3.2.3 and 6.5.1.3. Optical equipment alignment drift or partial plugging of orifices is discussed in subsection 6.5.2. Given that all these items are properly addressed, the final item listed above is probably the greatest source of error in the measurements. This error can be minimized by analyzing sample volumes that are as large as practical.

7.0 RECORDS

Records generated as a result of this DP are entries in laboratory notebooks or attachments to laboratory notebooks. The documentation should consist of any applicable items identified in Section 6.0, including completed Flow Cytometer Configuration sheets (Attachment 1) and printouts of signal intensity histograms. Laboratory notebooks should be kept in accordance with QP-03.5.

All records should be submitted to the Records Processing Center in accordance with QP-17.6.

8.0 ACCEPTANCE CRITERIA

Proper completion and submittal of the records described in Section 7.0 constitutes the acceptance criteria for this procedure.

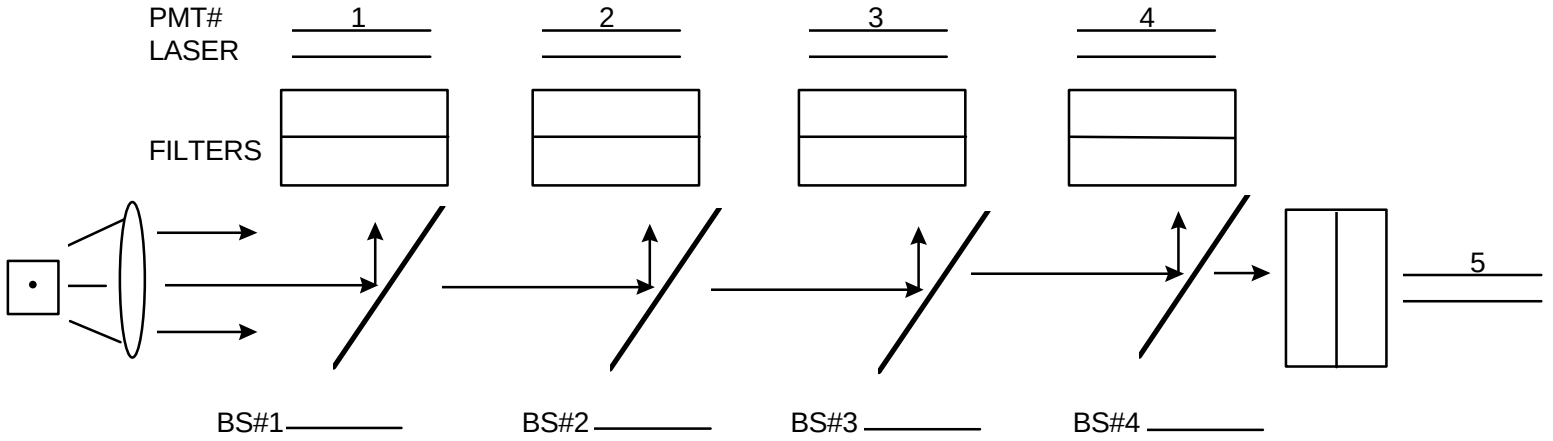
9.0 TRAINING REQUIREMENTS

Formal training is required for this DP. Training is documented in accordance with QP-02.7.

10. ATTACHMENTS

Attachment 1. Flow Cytometer Configuration Sheet (1 page).

DATA DISK | EXP. ID. | SORTER 1 DATE:



LASER	POWER	λ
1	mW	nM
2	mW	nM
3	mW	nM

DATA PARAMETERS + INITIAL SETTINGS

#	SIGNAL	LI	PMT	VOLTS	A/I	GAIN	DEC	SENS.	N.D.
1									
2									
3									
4									
5									
6									
7									
8									

Flow Cytometer Configuration Sheet